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ORIGINAL ARTICLE

Activation and modulation of antiviral and apoptotic genes in pigs infected with classical swine fever viruses of high, moderate or low virulence

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Abstract The immune response to CSFV and the strategies of this virus to evade and suppress the pigs' immune system are still poorly understood. Therefore, we investigated the transcriptional response in the tonsils, median retropharyngeal lymph node (MRLN), and spleen of pigs infected with CSFV strains of similar origin with high, moderate, and low virulence. Using a porcine spleen/intestinal cDNA microarray, expression levels in RNA pools prepared from infected tissue at 3 dpi (three pigs per virus strain) were compared to levels in pools prepared from uninfected homologue tissues (nine pigs). A total of 44 genes were found to be differentially expressed. The genes were functionally clustered in six groups: innate and adaptive immune response, interferon-regulated genes, apoptosis, ubiquitin-mediated proteolysis, oxidative phosphorylation and cytoskeleton. Significant up-regulation of three IFN- γ -induced genes in the MRLNs of pigs infected with the low virulence strain was the only clear qualitative difference in gene expression observed between the strains with high, moderate and low virulence. Real-time PCR analysis of four response genes in all individual samples largely confirmed the microarray data at 3 dpi. Additional PCR analysis of infected tonsil, MRLN, and spleen samples collected at 7 and 10 dpi indicated that the strong

induction of expression of the antiviral response genes chemokine CXCL10 and 2'-5' oligoadenylate synthetase 2, and of the TNF-related apoptosis-inducing ligand (TRAIL) gene at 3 dpi, decreased to lower levels at 7 and 10 dpi. For the highly and moderately virulent strains, this decrease in antiviral and apoptotic gene expression coincided with higher levels of virus in these immune tissues.

Introduction

Classical swine fever virus (CSFV), a member of the genus *Pestivirus*, family *Flaviviridae*, causes a severe disease of pigs that is characterized by fever, leucopenia and hemorrhage [65, 66]. Apoptosis has been demonstrated to be the cause of lymphocyte death [58, 66], but the precise mechanism by which CSFV causes apoptosis is still unknown.

Innate immunity is a critical arm of pre-existing host defence that functions to eliminate foreign pathogens immediately after infection and subsequently directs the adaptive immune response. Interferons (IFNs) are key components of innate immunity. Through different signal-transduction pathways, IFNs can promote the expression of dozens of genes responsible for executing IFN-induced anti-viral and anti-proliferative effects (reviewed in Ref. [30]).

The best-characterized IFN-inducible components of the antiviral response are the dsRNA-dependent protein kinase R (PKR, alias eukaryotic translation initiation factor 2- α kinase 2), the 2'-5' oligoadenylate synthetases (OASs), the Mx proteins and the nitric oxide system (NO). IFNs can control apoptosis by inducing a pro-apoptotic state in uninfected cells. For example, IFN has been shown to influence the sensitivity of cells for apoptosis by

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inducing expression of both the ligand and the receptor of FAS and TRAIL [71, 73]. In addition, IFNs can control the T helper immune system by inducing a broad spectrum of chemokines and cytokines such as CXCL10, which induce the Th1 immune response [18].

Viruses employ numerous evasive strategies to impair antiviral responses. In the case of CSFV, only a few viral strategies have been identified. The CSFV Npro protein has been described as a suppressor of IRF3 function in the IFN expression pathway in infected cells [3, 7, 17, 42, 55, 56, 60] and has been shown to interact with I κ B α , the NF- κ B inhibitor [23]. In addition, in CSFV-infected macrophages, NO production is suppressed by up-regulation of arginase activity [75]. However, those viral mechanisms only partially explain the immune suppression caused by CSFV. Therefore, CSFV most likely uses several other strategies to impair different immune pathways, e.g., the function of antigen-presenting cells, the regulation of apoptosis, antiviral responses and the complement system. These mechanisms have not yet been investigated for CSFV, and most of the current knowledge has been gained from *in vitro* experiments.

In this present study, we used micro-array and PCR analysis to investigate the transcriptional response to replication of CSFV in relevant immune organs of pigs. Pigs were infected with three different laboratory-derived variants of CSF virus strain Brescia [36], which were characterized as highly virulent, moderately virulent, and avirulent in earlier animal experiments [69]. However, because the avirulent strain did cause some fever and leucopenia, we will refer to it in this paper as low virulent. We identified viral modulations of IFN-inducible antiviral, apoptotic and chemotactic genes. To our knowledge, these data represent the first *in vivo* gene expression study that describes antiviral responses to CSFV.

Materials and methods

Study design

An animal experiment was carried out with four groups of nine pigs each. Three groups were inoculated with the highly, moderately or low virulent CSF strain, while the fourth group was left untreated. In each group, three piglets were euthanized at 3, 7 and 10 days post-inoculation (dpi) for the collection of tissue samples.

Animals

Thirty-six 6-week-old piglets were obtained from a conventional herd that was free of pestiviruses. Groups of four piglets of the same sex and originating from the same litter

were selected from the herd. From each group of four, one piglet was randomly assigned to one of the four treatment groups.

Inoculation

On day 0, the piglets of groups 1–3 were inoculated intranasally with 2 ml virus suspension (1 ml per nostril) containing 10^5 TCID₅₀ per ml of infectious virus, as determined using SK6 cells. The viruses used were, respectively, CoBrB (highly virulent: HV), C1.1.1 (low virulent: LV) and C1.1.1/CoBrB (moderately virulent: MV). Virus C1.1.1 is a cell-adapted variant of a Brescia strain with low virulence, whereas CoBrB (CoBrB 476S) is the virulent variant. Sequence analysis showed 29 nucleic acid mutations in C1.1.1, resulting in nine amino acid substitutions, compared to the sequence of CoBrB [36]. C1.1.1/CoBrB is a chimera from C1.1.1 and CoBrB, essentially containing the structural proteins from C1.1.1 and the non-structural proteins from CoBrB [69]. The control group was inoculated with 2 ml PBS instead.

Observation and scoring

From 3 days before the inoculation until they were euthanized, the animals were observed on a daily basis for clinical symptoms, and their rectal temperature was measured. A list of CSF-related clinical symptoms (lack of appetite, diarrhoea, lethargy, inability to stand up, trembling) was used and scored as either present or not present. The scores for each pig were added up to a total score per day.

Leucocyte count

EDTA blood samples were taken on days 0, 1, 2, 3, 4, 7, and 10 from all pigs that were still alive. White blood cell (leucocytes) and thrombocyte counts were performed using a Medonic[®] CA 620 coulter counter (Boule Medical AB, Stockholm, Sweden). Leucopenia was defined as $<8 \times 10^9$ cells/l blood, and thrombocytopenia as $<200 \times 10^9$ cells/l blood [12].

Post-mortem sampling

In each group, the first three piglets were euthanized on the first day on which the rectal body temperature was $>40^\circ\text{C}$. At 7 and 10 dpi, another three piglets were euthanized in each group. Post-mortem tissue samples were collected from the tonsils, spleen and median retropharyngeal lymph node (MRLN). They were snap-frozen in liquid nitrogen and stored at -80°C until total RNA was isolated from these samples.

Isolation of total RNA

From 0.5 g of tissue, total RNA (DNase-free) was isolated using TRIzol[®] reagent (Invitrogen) as described recently [50]. The yield and purity of the RNA was calculated from measurements of the extinction at 260 and 280 nm. The integrity of all RNA samples was checked by analyzing 0.5 µg of RNA on a 1% (w/v) agarose gel. After ethidium bromide staining, the gel was scanned to calculate the 28S/18S peak ratio (volume 28S over volume 18S) for each RNA preparation. RNA with a ratio >2 was considered of adequate quality to be used for real-time PCR and microarray analysis. For all RNA preparations, a 28S/18S ratio of >2 was observed. A part of each RNA preparation was used to prepare RNA pools for microarray analysis.

Microarray analysis

For microarray analysis a homemade porcine cDNA microarray containing 2,928 probes from a jejunum EST library [50] plus 2,880 probes from a spleen EST library was used. For production of this spleen EST library, spleens were collected from the same four 12-week-old pigs from which the jejunal mucosal scrapings were obtained for the preparation of the jejunum EST library [50]. A total of 2,688 probes (ESTs) were generated from total RNA isolated from pooled spleen tissue ($n = 4$ pigs). In addition, 192 probes were generated from RNA isolated from in vitro ConA/LPS-stimulated spleen cells derived from the above-described pooled spleen tissue. Together with the jejunum and spleen probes, an additional 192 cDNA probes selected from Marc1 and 2 EST libraries [24], and probes coding for porcine cytokines (IFN- γ , TNF- α , GM-CSF, IL-2, 4, 6, 8, and 10) and lung surfactant proteins SFTPA and SFTPD were spotted in quadruplicate on Corning UltraGAPS slides [31].

Dual-colour hybridization of slides was performed using an RNA MICROMAX TSA labelling and detection kit (Perkin-Elmer, Zoetermeer, The Netherlands) as described earlier [50]. Briefly, a biotin (BI)-labeled cDNA target was prepared from 1 µg of RNA template from an uninfected RNA pool, and a fluorescein (FL)-labeled cDNA target was prepared from 1 µg of RNA template from an infected RNA pool. Labelled cDNAs were simultaneously hybridized to a microarray slide and detected with Cy5 (biotin) and Cy3 (fluorescein), respectively. For each comparison, a second hybridization experiment was performed in which the BI and FL labels were reversed (dye-swap). The slides were scanned for Cy5 and Cy3 fluorescence in a Packard Bioscience BioChip Technologies apparatus (ScanArray Express, Perkin-Elmer). Scan images were processed with GenePix Pro 5.0 (Molecular Devices, Apeldoorn, The Netherlands), automatically gridding the spots, and

measuring spot intensities. In addition, spots with irregular shape were eliminated manually. An intensity-dependent normalisation was performed (blank-specific background correction; Lowess fit function with a fraction of 0.2 on all data points) using a customized version of the statistical software package R [74]. Significantly differentially expressed spots with a ratio of >3.3 or <0.30 (M -value of <-1.73 or >1.73; $M = \log_2(\text{Cy5/Cy3})$) and a p -value <0.05 were selected manually from normalized data reports. A probe was considered differentially expressed when at least five out of the eight spots present on the two dye-swaps passed these selection criteria. For each probe, the mean ratio of differential expression (infected over uninfected) of replicate spots ($n \geq 5$ and ≤ 8) is presented. Probes that were yet not annotated were sequenced as described previously [50].

Microarray comparisons

For each type of tissue, an RNA pool was prepared from total RNA, isolated from three pigs originating from the same infected group. At 3 dpi, these three piglets were selected from the HV and MV groups on the basis of early signs of fever (i.e., highest rectal temperature), and randomly from the LV group (see above). In three separate dye-swap hybridization experiments, mRNA expression levels in infected spleen, tonsil, and lymph node pools were compared to levels in RNA pools prepared from homologue tissue collected from the nine uninfected (control) pigs. Performing these tissue-specific microarrays for each virus strain (HV, MV, LV) resulted in nine data sets of probes hybridizing differentially with a fold change (ratio infected over uninfected) of <0.30 or >3.3 (p -value <0.05). Sequences of selected probes were annotated after blastn or (t)blastx analysis (when not yet annotated).

Real-time PCR for CSFV

For the detection of CFSV, PCR assays were performed using a LightCycler instrument with red dye, using the RNA Master Hybprobes kit (Roche Applied Science, Mannheim, Germany) [70]. C_t values were used to express the amounts of virus in a semi-quantitative way.

Real-time PCR for gene expression

Gene-specific primers were developed from mRNA reference sequences (pig, cattle, human) found in sequence databases (NCBI, KEGG). These reference sequences were selected by homology search with EST sequences obtained from the library probes. Primers were designed within the open frame of the protein encoded by the gene of interest and also, when possible, in different exons in order to

prevent amplification of genomic DNA. The primer sequences are listed in Table 1.

Randomly primed reverse transcription reactions were carried out using the Transcriptor first-strand cDNA synthesis kit (Roche Applied Science, Mannheim, Germany) with approximately 200 ng of RNA as template and 60 μ M hexamer primers. The produced cDNA was stored at -20°C until use. Real-time PCR tests were carried out with a LightCycler instrument and a LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Science, Mannheim, Germany). The PCR conditions are listed in Table 1. The relative concentration of mRNA was calculated by extrapolation on a standard curve prepared from dilutions of an RT reaction prepared from a reference RNA sample. The relative concentration of 18S and beta-actin were determined once for each cDNA sample and used to normalize concentrations of all other genes tested from the same cDNA sample. Established quantities of 18S rRNA and beta-actin showed essentially no differences among any of the individual RNA samples (18S RNA average relative concentration 1.15 ± 0.30 ; beta-actin average relative concentration 0.54 ± 0.026).

Results

Clinical parameters after infection with CSFV strains

Pigs infected with the HV and MV virus strains developed fever (above 40°C) around 2–3 dpi together with a drop in WBC count and, this was then followed by acute clinical signs beginning 4 dpi (Fig. 1). Although the drop in WBC's and fever for LV-infected pigs coincided with those of MV and HV-infected pigs, WBC concentrations and rectal temperatures reached normal levels at 7 dpi. Moreover, the moderate clinical signs observed in these pigs dramatically declined from 8 until 10 dpi. These

results indicated that LV-infected pigs quickly recovered from the infection. The pigs from the control group showed normal clinical parameters during the whole animal experiment (Fig. 1).

Gene expression analysis

Messenger RNA levels in CSFV-infected immune organs at the port of entrance (tonsil and medial retropharyngeal lymph node) and in the spleen at 3 dpi were compared to levels in homologous tissues from uninfected pigs for all three virus strains (LV, MV, and HV), using the home-made cDNA microarray prepared from spleen and jejunum tissue. In these nine microarray comparisons, a total of 44 different mRNAs (genes) hybridized differentially with a ratio (fold change; infected over uninfected) of <0.30 or >3.3 (p -value <0.05). Messenger RNA levels were increased significantly for 33 genes and reduced significantly for 11 genes. In Table 2, the ratio of differential expression calculated from the two dye-swap slides is presented. Based on literature search and data mining, a tentative function was assigned for the genes identified by blast analysis. With the probes coding for IFN- γ , TNF- α , GM-CSF, IL-2, 4, 6, 8, and 10, the fluorescence intensity was never higher than the background threshold.

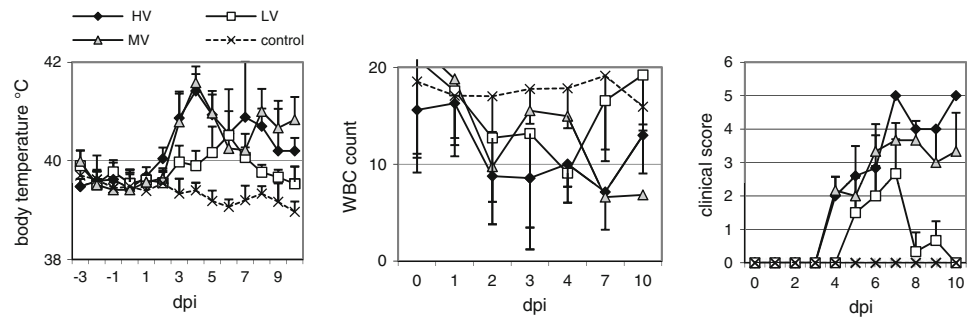
Data mining and functional clustering

Based on their function, genes identified by microarray analysis were clustered in six groups (Table 2): (1) genes related to apoptosis (Apopt.), (2) IFN- α/β - or γ -induced genes (IFN), (3) genes related to the adaptive and innate immune response, including antigen presentation (innate/adapt. Immune-AP), (4) genes related to the proteasome pathway, i.e., ubiquitin-mediated proteolysis (UBM), (5) components of, or proteins associated with the cytoskeleton

Table 1 Gene specific primer sequences and PCR conditions

Gene	Acc. number	Forward primer	Reverse primer	T _m (°C)	Reference
OAS2	AY288913	ACAGTCTTGAGGGGCAACTCTGA	GCTGGCTTTCATCATATCCAAGGA	60	De groot et al. (2005)
CXC10	AY789646	TGCCCACATGTTGAGATCAT	CGGCCCATCCTTATCAGTAG	60	
TRAIL	NM_001024696	CAACAAGGCATTTCCTCACCT	CCAGCTCTCCATTTCCTCAAG	60	
IFN alpha	NM_214393	TACTCAGCTGCAATGCCATC	TCTGTGCTGAAGAGCTGGAA	58	
18S RNA	AF102857	GTTCAAAGCAGGCCCGAG	CGCCGCCGCATCGCCA	57	
Beta actine	AY550069.1	GGCATCCTGACCCTCAAGTA	GGGTCATCTTCTCACGGTTG	58	
C4-bp	NM_213942	CTCTGGTTGGAGAGGACAGA	GCTCACAGTCTTCGGGGTA	60	

PCR conditions: 3 mM magnesium chloride and 0.5 mM for primer pair. The cycling PCR conditions consisted of 1 cycle at 95°C for 5', followed by 45 cycles (95°C for 5', annealing temperature for 5', 72°C for 12') and 1 three-segment cycle of product melting

Fig. 1 Body temperature, WBC and clinical score average data

(cytoskeleton), and (6) genes involved in oxidative phosphorylation, including oxygen transporters (Ox-P).

Up-regulated genes related to apoptosis included pro-apoptotic genes such as TRAIL, which leads to caspase-dependent cell death (reviewed in Ref. [64]) and the gene for cathepsin C, a protease responsible for the proteolytic maturation of granzyme [51], which induces apoptosis through activation of caspase and the Bid protein [8, 11]. Anti-apoptotic genes were also induced, such as MCL1 (alias BCL2-related), which blocks apoptosis mediated by ER- Ca^{2+} release and mitochondrial Cyt-*c* release [14], and is also involved in the regulation of redox- and oxidant-mediated apoptosis [32, 43]. In addition, genes related to glutathione metabolism (GLRX, GCLM), which also prevent redox- and oxidant-mediated apoptosis in the ER and mitochondria, were up-regulated [32]. Calbindin, a calcium-binding protein suppresses apoptosis by inhibiting caspase 3 activity [5]. Although PARP genes are well established as cell survival factors contributing to DNA repair, recent work has indicated that they also induce cell death via the p53 signalling pathway and via the intrinsic pathway [16, 33].

IFN-regulated genes were all up-regulated. These genes included IFN- γ -induced genes AIF-1, IFI 16, GBP-2, and MOP-5, and genes induced by IFN- α/β : IFI44, CXCL10, and OAS2. CXCL10 (alias IP10) is a pleiotropic cytokine that stimulates monocytes, natural killer and T cell migration [18]. The antiviral enzyme OAS2 catalyses the synthesis of oligoadenylate chains in response to dsRNA binding, which acts as an activator of RNase L [61]. Active RNase L degrades viral and cellular RNAs, leading to inhibition of cellular protein synthesis and impairment of viral replication (reviewed in Ref. [62]). The proteins AIF1, IFI16, GBP2, MOP5, and IFI44 are involved in cell growth, cellular defence and cell proliferation. Note that the up-regulation of the IFN- γ -induced genes AIF1, IFI16, and GBP2 in the MRLN of LV-infected pigs at 3 dpi was not detected in pigs infected with the MV and HV strains. Interestingly, it has been reported that expression of AIF1 in vascular muscle cells is induced in response to arterial injury, a phenomenon also observed in CSFV pathogenesis [27], and

IFI16 inhibits vascular tube morphogenesis and proliferation of primary endothelial cells [52].

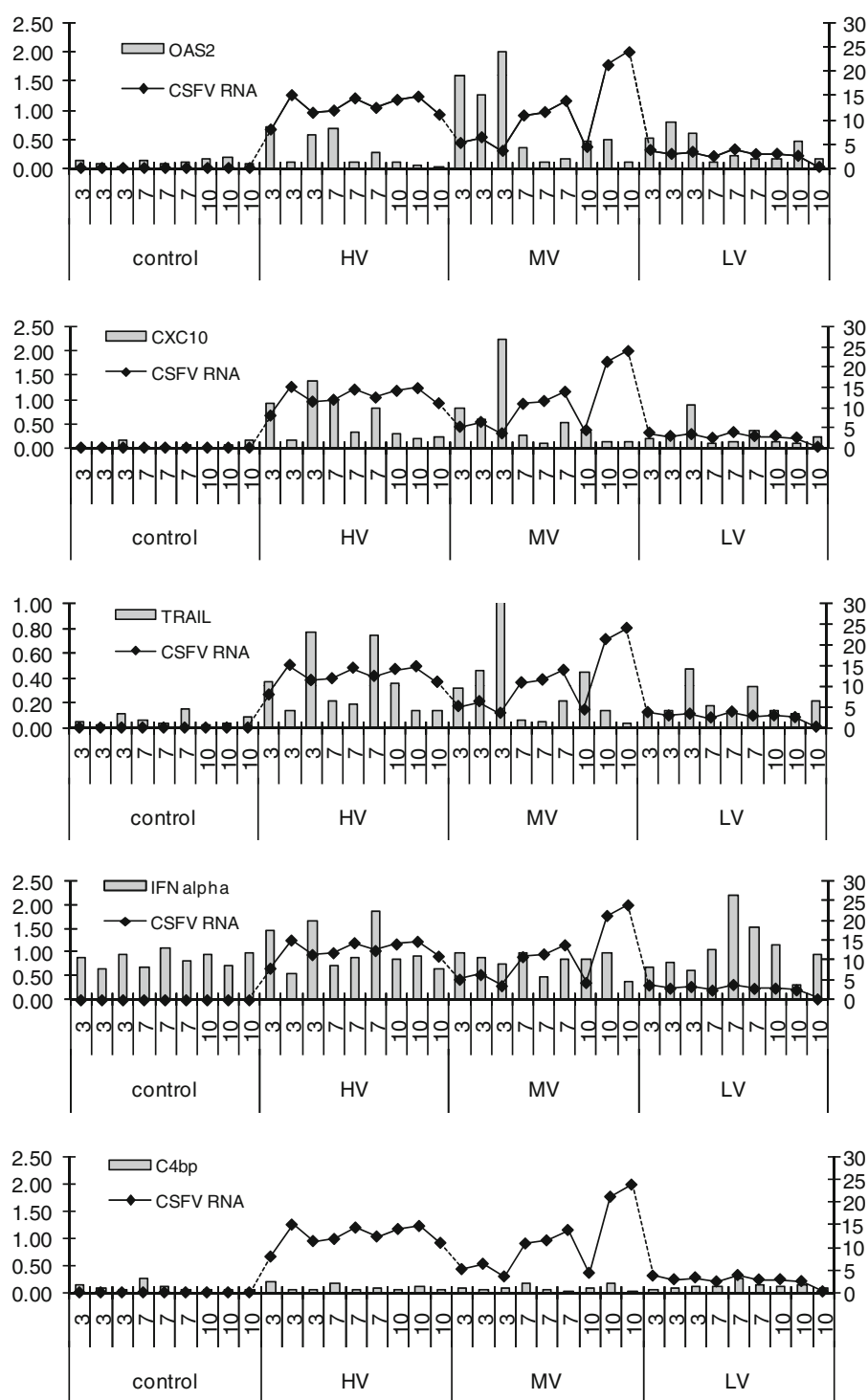
Up-regulated genes involved in the innate/adaptive immune response include MHC1 genes such as SLA-1 and B2M, the natural killer surface receptor CD16b and genes such as immunoglobulins (J, lambda, kappa chains, heavy, light chains, FC fragment), which are ubiquitously expressed on the surface of B and T lymphocytes. The gene C4b was down-regulated. C4bp is a regulatory component (inhibitor) of the classical complement cascade [9] but also acts as an activator of B cells (by binding to CD40) [15, 49] and a protein that helps with the removal of apoptotic cells by binding to protein S [10, 68].

Up-regulated genes involved in ubiquitin-mediated proteolysis included a potential ubiquitin ligase (HERC6) and components of the proteasome (UBC and Psma6).

Most of the genes found to be down-regulated were structural components of the cytoskeleton (ACTA 2 and TPM1), genes involved in oxygen binding and transport (alpha-beta globin and ferroportin-1), or constituents of the mitochondrial respiratory chain (oxidative phosphorylation) (CytB, ND6, ND4L and E-NPP 3).

RT-PCR studies

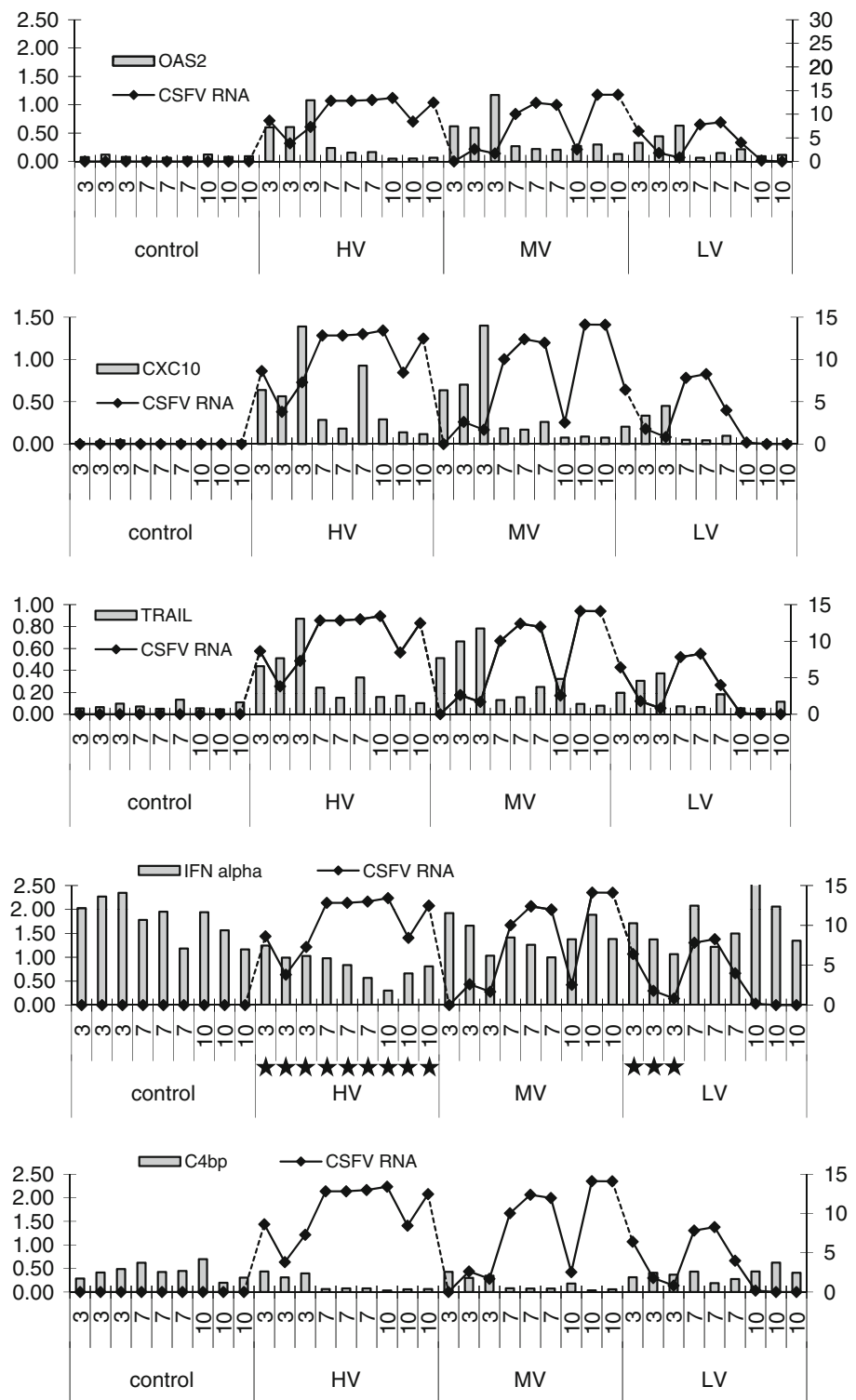
Three up-regulated anti-viral genes (OAS2, CXCL10, TRAIL) and a gene (C4bp) that was down-regulated specifically in the MRLN were selected for real-time PCR studies. The expression levels of these genes were determined in all tissue sample collected from all individual pigs at 3, 7 and 10 dpi. In addition, IFN α expression and CSFV replication were also investigated in all these samples (Figs. 2, 3, 4). In agreement with the microarray data, a strong up-regulation of anti-viral gene expression was observed in all three immune tissues collected from LV-, MV- and HV-infected pigs at 3 dpi. At 7 and 10 dpi, this high level of expression dropped to lower levels, and in some pigs to the (normal) levels observed in uninfected pigs. In contrast, PCR results obtained for C4bp expression in MRLNs of LV- and HV-infected pigs at 3 dpi (Fig. 4) did not match with the data of the microarray, most likely due to a naturally occurring high inter-animal variation of C4bp

Fig. 2 Gene expression in tonsil

expression in the MRLN (see control animals in Fig. 4). However, similar to what was observed for the MV strain, C4bp expression was significantly reduced at 7 and 10 dpi in MRLNs and spleens of most HV-infected pigs (Figs. 3, 4).

In all three immune organs of most MV- and HV-infected pigs, a high level of virus seemed to be associated with a low level of up-regulation of OAS2, CXCL10,

and TRAIL. For LV-infected pigs, this inverse correlation between viral replication and anti-viral gene expression was also apparent in spleens and MRLNs obtained at 3 dpi and in spleens obtained at 7 dpi. However, in spleens and MRLNs collected from LV-infected pigs at 10 dpi, hardly any (or no) viral replication could be detected and no significant up-regulation of these anti-viral

Fig. 3 Gene expression in spleen (* p -value <0.05)

genes was observed. These results suggested that regulation of OAS2, CXCL10, and TRAIL gene expression in these immune organs is tightly linked to replication of CSFV. Interestingly, an inverse correlation between virus replication and IFN α gene expression was

also observed in immune organs of a few infected pigs (marked with an asterisk in Fig. 4, p -value <0.05). Because IFN α gene expression in uninfected pigs was very capricious (see control animals in Fig. 4), more data are needed to draw conclusions about this.

Table 2 Differential expressed mRNAs detected by microarray analysis at 3 dpi

ID (#)	Fold change ^a						Blastn or tblastx ^b						Tentative function (functional cluster) ^c
	Spleen			Lymph node			Tonsil			Gene name or tentative annotation (T[H]C)	Acc. number	E-value (nt)	
	LV	MV	HV	LV	MV	HV	LV	MV	HV				
Higher in infected													
CSF-1	-	-	-	4.0	-	-	-	-	-	Allograft inflammatory factor 1 (AIF1)	<u>XM_001490247</u>	4E-154 (474)	Growth-suppressing (IFN- γ)
CSF-2	-	-	-	3.8	-	-	-	-	-	IFN-gamma-inducible protein 16, transcript variant 1 (IFI16), mRNA	<u>ref XM-001170179.1 </u>	4E-47 (656)	Growth-suppressing (IFN- γ)
CSF-3 (2)	-	-	-	4.2	-	-	-	-	-	Guanylate binding protein 2 (interferon-inducible) (GBP2), mRNA	<u>ref NM-004120.</u>	1E-123 (711)	Signal transduction/antiviral effect (IFN- γ)
CSF-4	-	-	-	3.5	4.6	-	-	-	-	Similar to dendritic cell-derived IFN γ -induced monocyte protein 5 (MOP5)	<u>pig TC265382</u>	2E-54 (432)	Metal dependent phosphohydrolase (IFN- γ)
CSF-5	-	-	-	3.5	-	4.8	-	-	-	Polyubiquitin, transcript variant 12 (UBC), mRNA	<u>XM_874550.3</u>	0 (790)	Component proteasome (UMP)
CSF-6	-	5.3	5.0	-	-	-	-	-	-	Proteasome (prosome, macropain) subunit alpha type, 6 (Psm α 6) mRNA	<u>NM_002791</u>	0 (658)	Component proteasome (UMP)
CSF-7 (3)	5.6	5.7	6.1	5.3	5.1	-	5.3	5.4	6.0	HECT domain and RCC1-like domain-containing protein 6 (HERC6)	<u>Pig TC273610</u>	2.9E-121 (715)	Potential ubiquitin ligase (UMP)
CSF-8 (2)	4.6	5.7	5.9	4.3	5.7	-	3.9	5.4	4.6	Similar to poly (ADP-ribose) polymerase family, member 14, mRNA (BAL2)	<u>BC120371</u>	5E-179 (626)	DNA-damage induced/cell survival (UMP/Apopt.)
CSF-9 (2)	-	5.5	6.0	-	-	-	-	5.7	5.1	Similar to HUMAN (Q9H0J9) Poly [ADP-ribose] polymerase 12 (PARP12)	<u>pig TC260164</u>	2E-101(752)	DNA-damage induced/cell survival (UMP/Apopt.)
CSF-10	-	4.7	4.9	-	-	-	-	-	-	Poly (ADP-ribose) polymerase family, member 9 (PARP9)(BAL1), mRNA	<u>NM_001076828.</u>	2E-165 (817)	DNA-damage induced/cell survival (UMP/Apopt.)
CSF-11	7.2	6.8	9.9	6.3	-	-	-	-	-	Similar to HCV-associated microtubular aggregate protein p44 (IFI44)	<u>THC2463234</u>	2.9E-23 (534)	Antiproliferative activity (IFN- α/β)
CSF-12	39	49	38	40	39	41	27	45	38	CXC-like protein gene, complete cds (CXCL10/IP10)	<u>AY894692.</u>	0 (533)	Pleiotropic cytokine (IFN- α/β)
CSF-13	21	23	18	14	13	13	18	25	24	2'-5' oligoadenylate synthetase 2 (OAS2)	<u>ref NM-001031796.1 </u>	2E-136 (309)	dsRNA mediated antiviral response (IFN- α/β)
CSF-14	6.0	7.7	7.1	3.7	5.2	-	-	5.8	4.6	TNF-related apoptosis-inducing ligand (TRAIL) mRNA	<u>ref NM-001024696.1 </u>	0 (1004)	Cytokine TNF ligand family (Apopt.)

Table 2 continued

ID (#)	Fold change ^a						Blastn or tblastx ^b						Tentative function (functional cluster) ^c
	Spleen			Lymph node			Tonsil			Gene name or tentative annotation (T[H]C)	Acc. number	E-value (nt)	
	LV	MV	HV	LV	MV	HV	LV	MV	HV				
CSF-15	-	5.8	6.5	-	6.0	10.2	-	7.2	4.4	EST weakly similar to HUMAN (Q7Z5U7) cathepsin C (CTSC)	<u>human/THC2686463</u>	0.22 (276)	Lysosomal cysteine proteinase/ requires Cl ⁻ (Apopt.)
CSF-16	-	4.0	6.0	4.4	-	-	-	-	-	Similar to NADH-ubiquinone oxidoreductase chain 1 (NADH1)	<u>reflXNM-001113286.11</u>	6e-81(374)	Subunit mitochondrial complex I (Apopt.)
CSF-17	-	-	-	4.4	4.0	-	-	-	-	Myeloid cell leukemia sequence 1 (MCL1)(BCL2-related)	<u>reflNMM-021960.31</u>	3E-136 (671)	Cell survival/anti-apoptotic (Apopt.)
CSF-18	12	18	13	12	9.4	-	11	15	14	Glutamate-cysteine ligase, modifier subunit (GCLM), mRNA	<u>reflNMM-199845.11</u>	5E-29 (244)	Key enzyme glutathion metabolism (Apopt.)
CSF-19 (3)	-	5.6	6.3	4.0	5.8	8.7	-	6.4	5.0	Glutaredoxin (thioltransferase) (GLRX), mRNA	<u>reflNMM-001037604.11</u>	7e-51 (611)	Cell redox homeostasis/ deglutathionylation (Apopt.)
CSF-20	-	5.1	6.6	5.9	5.9	-	-	-	-	Calbindin D-9 k mRNA	<u>NM_214140.</u>	1E-174 (516)	Calcium binding and transport activity (Apopt.)
CSF-21	3.6	-	-	-	-	-	-	-	-	Sus serofa MHC class I antigen 1 (SLA-1), mRNA	<u>reflNMM-001097431.11</u>	1E-86 (255)	Antigen presentation MHC I (Innate immune AP)
CSF-22	-	-	-	-	-	-	-	3.5	-	Beta-2-microglobulin (B2 M), mRNA	<u>reflNMM-004048.21</u>	1E-26 (476)	Antigen presentation MHC I (Innate immune AP)
CSF-23	-	-	-	-	-	-	-	3.9	-	Fc fragment of IgG, low affinity IIIb, receptor (CD16b)	<u>reflNMM-214391.11</u>	0 (794)	Natural killer cell mediated cytotoxicity (Innate Immune-AP)
CSF-24 (7)	-	-	-	4.5	4.2	-	-	-	-	Immunoglobulin J chain	<u>reflXNM-001105782</u>	8e-98 (348)	Joining IgM and IgA monomers (Adapt. Immune-AP)
CSF-25 (2)	-	-	-	3.8	3.7	-	-	-	-	Immunoglobulin lambda-chain (CD179b antigen)	<u>reflXNM-850728.11</u>	2e-96 (677)	Pre- B or T cell receptor (Adapt. Immune-AP)
CSF-26 (5)	-	-	-	3.9	4.2	-	-	-	-	Kappa light chain mRNA	<u>reflNMM-001093783.11</u>	1E-77 (494)	Pre- B or T cell receptor (Adapt. Immune-AP)
CSF-27 (2)	-	-	-	3.7	-	-	-	-	-	Immunoglobulin mu heavy chain constant region, mRNA	<u>gbIS42881.11</u>	0 (661)	Pre- B or T cell receptor (Adapt. immune-AP)
CSF-28 (2)	-	3.8	-	-	-	-	-	-	-	Placenta-specific 8 (PLAC8), mRNA	<u>NM_016619.</u>	3e-59 (330)	Expressed in neutrophils/cell survival (Apopt. ?)
CSF-29	-	3.8	-	-	-	-	-	-	-	Sterile alpha motif domain containing 9 (SAMD9)	<u>XM_001492397</u>	2e-170 (595)	Cell proliferation (Apopt. ?)
CSF-30	-	-	4.6	-	-	-	-	-	-	Similar to frizzled 5 (LOC710796), mRNA (FZD5)	<u>reflXNR-012291.11</u>	2e-12 (418)	Receptor for Wnt signaling proteins
CSF-31	4.5	4.5	4.5	5.3	4.7	-	-	5.5	-	EST weakly similar to myelin expression factor 2 (MyEF2)	<u>human/THC2606750</u>	0.00012 (547)	Myocyte transcription enhancer
CSF-32	-	5.5	6.0	-	4.9	-	-	-	-	Ribosomal protein L9 homologue (RPL9)	<u>XM_001149849</u>	7E-160 (790)	Structural component ribosome

Table 2 continued

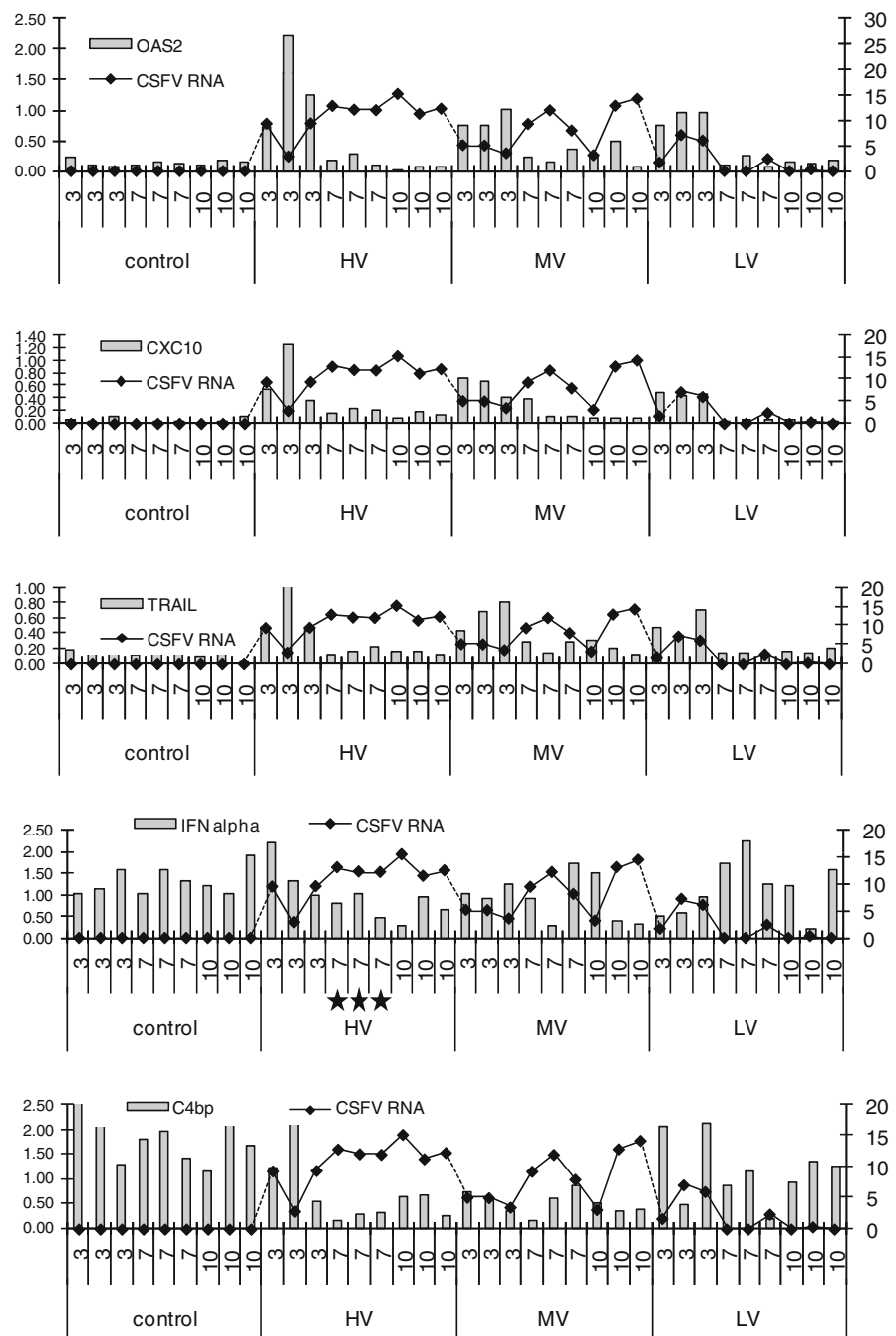
ID (#)	Fold change ^a						Blastn or tblastx ^b	Gene name or tentative annotation (T[H]C)	Acc. number	E-value (nt)	Tentative function (functional cluster) ^c			
	Spleen			Lymph node								Tonsil		
	LV	MV	HV	LV	MV	HV						LV	MV	HV
CSF-33	-	4.1	4.9	-	-	-	-	-	Chloride intracellular channel 5 (CLIC5), transcript variant 2, mRNA	ref NM-016929.3	5e-40 (564)	Voltage-gated chloride channel (cytoskeleton)		
Lower in infected														
CSF-34	-	0.16	0.18	-	-	-	-	-	Actin, alpha 2, smooth muscle, aorta (ACTA2), mRNA	ref NM-001034502.1	9e-76 (237)	Component cytoskeleton (cytoskeleton)		
CSF-35	-	0.20	0.22	-	-	-	-	-	Tropomyosin, transcript variant 25 (TPM1), mRNA	ref XM-860199.1	5e-167 (623)	Component cytoskeleton (cytoskeleton)		
CSF-36 (7)	-	0.29	-	0.12	0.13	0.16	-	-	Tblastx; mRNA clone:LVRM10070B07 homologue to <i>B. taurus</i> elastin	dbj AK233022.1	0 (613)	Extracellular fibre		
CSF-37 (4)	-	-	-	0.09	0.07	0.11	-	-	Complement component 4 binding protein, alpha (C4BPA)(APOR), mRNA	ref NM-213942.1	0.0 (537)	Complement cascades (innate and adapt immune-AP)		
CSF-38 (6)	-	-	-	0.14	0.17	-	-	-	Beta globin mRNA	ref XM-537902.2	5e-172 (565)	Oxygen transport (Ox-P)		
CSF-39	-	-	-	0.24	-	-	-	-	Hemoglobin alpha chain	ref NM-001077422.2	4e-117 (593)	Oxygen transport (Ox-P)		
CSF-40 (2)	-	-	-	0.25	-	-	-	-	Solute carrier family 40 member 1 (Ferroportin 1), mRNA	XM_001250723	6e-87 (388)	Cellular iron ion homeostasis (Ox-P)		
CSF-41 (3)	-	0.23	0.21	-	-	-	-	0.27	Cytochrome <i>b</i> (CYTB)	ref NP-008646.1	1E-54 (434)	Oxidative phosphorylation (Ox-P)		
CSF-42	-	0.29	0.29	-	-	-	-	-	EST homologue to NADH dehydrogenase subunit 4L (ND4L)	pig TC238572	1.0e-18 (468)	Oxidative phosphorylation (Ox-P)		
CSF-43	-	-	0.28	-	-	-	-	0.23	Blastx: NADH dehydrogenase subunit 6 (ND6)	ref NP-008645.1	3e-50 (524)	Oxidative phosphorylation (Ox-P)		
CSF-44	-	-	0.23	-	-	-	-	-	Ectonucleotide pyrophosphatase/phosphodiesterase 3 (ENPP3/CD203c)	ref NM-001075923	0.0 (670)	Hydrolysis of Flavin adenine dinucleotide (Ox-P)		

^a Fold change; ratio of differential expression (infected over uninfected) of library probes (ID). The number of library probes (#) aligning to an identical accession number and also hybridizing differentially is given in *parentheses* behind the ID. For these sets of identical probes an average fold change was calculated

^b DNA sequences of array probes were compared with the NCBI RNA reference sequence, NCBI non-redundant (nr), or the DFCI EST databases using blastn (or tblastx when indicated) and WU-BLAST 2.0 blast(n), respectively. Gene names or annotations of tentative consensus sequences (T[H]C), which scored the highest degree of homology, i.e., lowest *E*-value, are listed with their accession or T[H]C numbers (acc. number). The length of the probe-sequence (nt) compared is given in *parenthesis* behind the *E*-value. Based on data mining in the NCBI (Pub Med, Gene, OMIM, Unigene, conserved domain), HRPD, KEGG, Reactome, and BioCarta (pathway) databases a tentative function and/or process was assigned, and genes were clustered

^c Based on data mining in the NCBI (Pub Med, Gene, OMIM, Unigene, conserved domain), HRPD, KEGG, Reactome, and BioCarta (pathway) databases a tentative function was assigned and functional cluster: (Apopt.), related to apoptosis. (IFN) IFN- α/β or γ induced. (Innate/adapt. immune-AP) adaptive and innate immune response including antigen presentation. (UBM), related to the proteasome pathway, i.e., ubiquitin-mediated proteolysis. (Cytoskeleton) components of, or proteins associated with the cytoskeleton. (Ox-P), involved in oxidative phosphorylation and oxygen transport

Fig. 4 Gene expression in MRLN (* p -value <0.05)



Discussion

In the present study, we investigated the factors involved in the immune response of pigs during CSFV infection by determining gene expression profiles in immune organs. We demonstrate the extent of the porcine immune response against CSFV and the modulation of the host-signalling pathways by the virus. Besides the up-regulation of a cluster of IFN- γ -induced genes (AIF-1, IFI16, and GBP-2) in MRLN specifically induced by low virulent Brescia, no other pronounced qualitative differences in modulation of

host pathways were detected between the strains of different virulence tested here. This emphasizes the complex interplay between CSFV and its host, in which subtle differences in genetic background of the virus and subtle differences in host factors, e.g., age and breed [21, 48], are decisive for the course of this viral disease.

The gene expression profiles provided by microarray indicated that entry and replication of CSFV modulated every part of the porcine immune system: (1) innate immunity with its humoral and chemical barriers such as the complement system and inflammation, as well as

cellular barriers such as phagocytes, and (2) adaptive immunity, which involves B cells, killer T cells, and helper T cells.

Cytokines and chemokines such as IL6, IL8, TNF- α , IFNs, IL10 and IL12 have been demonstrated to be induced by CSFV [7, 38]. However, in the present study, CXCL10 is the most strongly up-regulated cytokine. CXCL10 (alias IP10) has been reported to be involved in effector T cell, monocyte and NK recruitment in response to infections with viruses involved in neurological disease, liver disease, heart disease and hemorrhagic fever disease [1, 18, 41, 44, 45]. It also has a pro-apoptotic effect via the p53-mediated (intrinsic) apoptotic pathway [76], which, according to our gene expression profiles, was also found to be activated by CSFV. This suggests that CXCL10 plays an important role in innate immunity through the recruitment of immune cells but also as a potential pro-apoptotic factor in CSFV disease.

The activation of TRAIL by CSFV confirms the conclusions of previous studies showing that programmed cell death is involved in the mechanism of lymphopenia during CSF [19, 66] and extends the list of death ligands known to be used by CSFV (TNF- α and FAS). Indeed, TRAIL functions as an antiviral protein [13, 72] by inducing cell death in infected cells via the extrinsic pathway but can also induce immunosuppression. It has been demonstrated that viruses such as HIV, measles virus, and cytomegalovirus infect DCs and induce TRAIL and TNF- α expression on their surface [64, 71]. Such DCs cannot present antigens and prime T cells for the generation of virus-specific CTLs. Instead, they kill interacting T, B and NK cells via the FAS and TRAIL/death-receptor pathway. This suggests that TRAIL plays a key role in the induction of apoptosis in uninfected cells and is also an immunosuppressor in CSF.

CFSV, by its replication in host cells, induced ER stress and oxidative stress, which intrinsically trigger apoptosis. As a countermeasure, several anti-apoptotic genes may have been activated, such as MCL1 (a gene related to Bcl-2), glutathione metabolism genes, and the gene for the Ca²⁺ regulation/transport protein calbindin. These observations are in agreement with previous *in vitro* studies with bovine virus diarrhoea virus, in which Bcl-2 gene expression was up-regulated in MDBK cells infected with a noncytopathic strain and down-regulated in these cells when infected with a cytopathic strain [6, 40]. This Bcl-2 down-regulation resulted in a reduced production of the antioxidant glutathione. Other viruses such as African swine fever virus or Epstein-Barr virus are known to regulate apoptosis by the synthesis of a viral protein homologous to Bcl-2 ([54], reviewed in Ref. [22]). Our results suggest that such anti-apoptotic gene activation, which may afford significant protection against virus-induced cell death, also may play a role in the pathogenesis of CSFV.

No genes involved in NO metabolism were found to be regulated by CSFV.

The results show IFN-response-gene activation, although no significant up-regulation of IFN genes was found. This could be explained either by very early and short IFN expression (before day 3) [35] or by a low level of IFN expression produced by plasmacytoid DCs, which were not inhibited by CSFV [2, 57, 67] and probably undetectable by microarray due to the relatively low concentration of these cells in whole tissue samples.

CSFV also affects the regulation of the complement system by shutting down the normal host complement inhibitor C4bp. Since the complement regulator proteins (C4bp, factor H and factor I) have been shown to be major targets for inhibition of complement by viruses [20, 25], this down-regulation could reflect a viral defence response. However, C4bp has been implicated in other functions such as B-cell activation and the removal of apoptotic cells and bacteria (reviewed in Ref. [10]). Therefore, further investigation is necessary for a better comprehension of the C4bp function in CSF.

Ubiquitin-mediated proteolysis by the proteasome, another pathway identified by the microarray, is involved in directing protein trafficking and degradation (reviewed in Ref. [46]). In CSFV infection, the proteasome has been shown to degrade interferon regulatory factor 3 (IRF3) after interaction with viral protein N-Pro [4, 60], leading to the interruption of the transcription of IFNs. In addition, it has been shown that various other viruses can stimulate ubiquitin-mediated degradation of regulatory factors for their own benefit, for example, to support evasion of the host immune system (e.g., MHC1 or STAT1 and 2, regulators of IFN expression; [26]), and to suppress apoptosis (e.g., the degradation of IAP, NFkB or Bcl-2; reviewed in Ref. [39]). Given that CFSV infection leads to immune suppression and apoptosis, the ubiquitin-proteasome is more likely to be used by CSFV to control, in addition of the IRF3, some of those crucial pathways. Anti-viral gene expressions (OAS2, CXCL10) and pro-apoptotic gene expression (TRAIL) showed a marked decrease when the virus replicated to a high level.

Although the WBC count is low in the animals at that stage of the disease (7 and 10 dpi), the depletion of T cells and DC cells does not by itself explain the decrease in gene expression. All of the gene data were normalized with 18S and beta-actin genes in order to eliminate this type of error. In addition, not all of the tested genes showed a decrease in gene expression. For example, MOP5 (data not shown) which is also an IFN-induced gene, showed no significant decrease in gene expression at 7 and 10 dpi.

This study also suggests that CSFV can modulate host-signalling pathways to enhance its persistence and survival. The exact mechanism of the modulation remains to

be elucidated. However, several strategies have already been identified to control those pathways. For example, poliovirus, reovirus and influenza A virus can inhibit OAS activation by viral dsRNA-protein binding (reviewed in Ref. [63]). Several RNA and DNA viruses block IFN signaling in order to inhibit multiple pathways simultaneously, such as cellular antiviral enzymes (OAS, PKR, Mx1), anti-inflammatory response (CXCL10), and death ligands (TRAIL, FAS, TNF- α ; reviewed in Ref. [53]). In addition to modulation of IFN production by Npro and Erns [28, 34, 37, 47], the pestivirus BVDV shows modulation of IFN signaling, such as PKR and MX activities, to establish a persistent infection [29, 59].

In conclusion, the functional analyses of gene expression show that most of the expressed genes contribute to anti-virus defense responses and pathogenesis of CSFV, while others reflect viral defense responses. Therefore, our findings can provide new insights into understanding the molecular basis of pathogenesis in CSFV pigs.

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